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Assessment of ROS production in the mitochondria of live cells

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Abstract

Production of reactive oxygen species in mitochondria plays multiple roles in physiology, and excessive production of ROS leads to the development of various pathologies. ROS in mitochondria are generated in various enzymes, mainly in the electron transport chain and it is important to identify not only the trigger, but also the source of free radical production. It is important to measure mitochondrial ROS in live, intact cells, because activation of ROS production could be initiated by changes in extramitochondrial processes which could be overseen when using isolated mitochondria. Here we describe the approaches which allow to measure production of ROS in the matrix of mitochondria in live cells. We also demonstrate how to measure kinetic changes in lipid peroxidation in mitochondria of live cells. These methods could be used for understanding the mechanisms of pathology in a variety of disease models and also for testing neuro- or cardioprotective chemicals.

Keywords: mitochondria, ROS production, lipid peroxidation, live cell imaging

Running Title: ROS production in mitochondria

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1. Introduction

Free radicals and reactive oxygen species (ROS) are produced in the cells in enzymatic and non-enzymatic reactions. The level of ROS production depends on the environment, rate of the metabolism and many other factors. Highly-producing ROS enzymes such as NADPH oxidase or xanthine oxidase generate ROS only at the time of activation. Mitochondrial electron transport chain (ETC), contrarily, is the source of ROS in live cells, which are constantly produced due to electron escape. The superoxide anion is the proximal ROS formed in the mitochondria, where it is rapidly converted to hydrogen peroxide by Mn-superoxide dismutase (SOD2) (Slot, Geuze, Freeman, & Crapo, 1986). Overproduction of hydrogen peroxide lead to increased levels of lipid peroxidation and is the cause for oxidative stress - implicated in many brain pathologies (Abeti et al., 2016; Angelova et al., 2015; Jiang et al., 2003). The rate of ROS production in the ETC of mitochondria depends on the metabolic state, presence of inhibitors, mutations and many others. The term mitochondrial ROS usually describes production of ROS in ETC, but mitochondria also generate ROS via other enzymes: monoamine oxidase (MAO), located on the outer membrane of mitochondria, and in enzymes of the tricarboxylic acid cycle in the mitochondrial matrix (Angelova & Abramov, 2016, 2018; Vaarmann, Gandhi, & Abramov, 2010).

Considering the high production of ROS in mitochondria and their involvement in energy metabolism, mitochondrial ROS have been suggested to affect the ageing process. Excessive ROS production is a trigger for the development of pathology in neurodegenerative disorders, specifically in Parkinson's disease, as well as in cardiac pathology, ischaemia/reperfusion injury and cancer (Abramov & Duchen, 2008; Abramov, Scorziello, & Duchen, 2007; Angelova & Abramov, 2018; Angelova et al., 2018; Angelova et al., 2015; Arber et al., 2017; Deas et al., 2016; Esteras, Rohrer, Hardy, Wray, & Abramov, 2017; Reeve et al., 2015). Thus the ability to measure mitochondrial ROS is important for our understanding of the mechanisms of any pathology and finding the ways for cell protection.

Mitochondria produce ROS both into the matrix of mitochondria and outside mitochondria. Moreover, despite that the proximal mitochondrial ROS, the superoxide anion, has a very short lifetime and is membrane impermeable, its conversion in the superoxide dismutase reaction to the more stable membrane-permeable hydrogen peroxide makes it possible to transfer ROS from the mitochondrial matrix to the cytosol. This is an important consideration as most of the measurements of ROS in live cells measure the rate of the oxidation of specific

compounds/probes with fluorophores in the matrix of mitochondria and very often ignore the ROS, released from the organelle.

Mitochondria, and cells in general, are protected by highly effective antioxidant systems and the increase of mitochondrial ROS production *per se* does not necessarily report on the occurrence of oxidative stress. Thus, we have observed higher levels of mitochondrial ROS production in primary glio-neuronal cultures isolated from brain of Kelch-like ECH associated protein 1 (Keap1)-knockdown mice compared to their wild-type counterparts (Kovac et al., 2015). Keap1 is the main negative regulator of transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2, gene name *NFE2L2*), the master regulator of the cellular redox homeostasis, which connects cellular redox with intermediary metabolism (Hayes & Dinkova-Kostova, 2014). Despite the increased levels in ROS production, Keap1-knockdown cells, which have constitutively high levels of Nrf2, are not under oxidative stress. The levels of reduced glutathione (GSH) are not significantly different in Keap1-knockdown than in wild-type glio-neuronal cultures (Holmstrom et al., 2013). This is because Nrf2 controls the expression of the enzymes that are responsible for the biosynthesis and regeneration of GSH and NADPH, including the xCT subunit of system xc–, which imports cystine into cells, the catalytic and modifier subunits of glutamate-cysteine ligase, which catalyses the rate-limiting step in the biosynthesis of GSH, glutathione reductase, which reduces GSSG to GSH, as well as the four principal NADPH-generating enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, and malic enzyme 1. In addition, the expression of thioredoxin 1, thioredoxin reductase 1, and sulfiredoxin 1, which reduce oxidized protein thiols, is also controlled by Nrf2. The apparent ‘paradox’ of high mitochondrial ROS under conditions of Nrf2 activation (and thus absence of oxidative stress) can be explained by the fact that the mitochondrial ROS production is linked to the rate of respiration, with higher rates of respiration leading to greater ROS production. Indeed, respiration is increased in Keap1-knockdown cells (Holmstrom et al., 2013). For assessment of oxidative stress, assessments of protein oxidation, levels of endogenous antioxidants (such as GSH) or lipid peroxidation must be conducted in combination with ROS measurements.

Fluorescence imaging of ROS in living cells provides a helpful tool to monitor production of ROS in more “native”, physiological conditions. Here, we describe quantitative methods for measuring the rate of ROS production in mitochondria of live cells using the specific indicators MitoSOX Red and MitoTracker Red CM-H2XRos. We also demonstrate a method

for the quantification of the rate of lipid peroxidation in mitochondria of permeabilised cells in pseudointracellular solution, using BODIPY™ 581/591 C11.

2. Materials

1. Glass coverslips or 30mm Petri dish with glass bottom.

2. Steel holding chamber, if class coverslips are used

3. Pluronic (Molecular Probes/ Invitrogen): prepare a 2% stock solution in DMSO.

4. MitoSOX Red (Molecular Probes, M36008) supplied in 10 x 50 µg vials. Dissolve the content of a vial by adding 13 µl fresh DMSO to prepare a stock solution of 50mM, and store at -20 °C (upright, protected from light) for up to 1 month.

5. MitoTracker Red CM-H2XRos (M-7512) supplied in 10 x 50 µg vials. Dissolve the content of a vial by adding fresh DMSO to the tube to prepare a stock solution of 1mM, and store at -20 °C.

6. BODIPY™ 581/591 C11 (ThermoFischer, D3861): add 200 µl fresh DMSO to the 1 mg tube before use, and store at -20 °C for up to 1 month.

7. HEPES-buffered salt solution (HBSS): 10 mM glucose, 156 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM HEPES; pH 7.35.

8. Permeabilization solution for measuring lipid peroxidation in mitochondria based on the "pseudo-intracellular" solution (IPS); (Abramov & Duchen, 2010) : 135 mM KCl, 10 mM NaCl, 20 mM HEPES, 5 mM pyruvate, 5 mM malate, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM EGTA, and 1.86 mM $CaCl_2$; pH 7.1 + 20µM digitonin (Sigma, D141);

9. Washing and imaging medium for lipid peroxidation – based on the IPS; pH 7.1 + 5 µM BODIPY™ 581/591 C11.

10. Inverted laser-scanning confocal microscope equipped with HeNe (514 nm or 561 nm wavelength) and Ar (488 nm wavelength) lasers and a heating chamber to maintain 37°C.

3. Methods

3.1 Preparation of the imaging material

1. The cell lines or primary cell cultures of interest should be grown on glass coverslips (VWR ECN 631-1584) with a certain diameter, conveniently fitting the measuring chamber. Preparation of the class cover slips encompasses baking at 250°C for 2 h, pre-treatment with 1M hypochloric or nitrous acid for 1 h and 3x wash with ddH_2O_2

2. The pre-treated class coverslips are placed in a 6-well plate and coated with Poly-L-lysine (Sigma, P4832), poly-D-lysine (Sigma, A-003-M), Laminin (Sigma, 11243217001), poly-L-ornithine (Sigma, P4957), collagen (Sigma, 5162) or gelatine (Sigma, 1.04070), following the specificity of the cells of interest, to ensure adhesion of the cell material to the glass for a stable time-course imaging.

3. In case of primary culture, the tissue is extracted and cut into small pieces in PBS buffer, thereafter transferred to trypsin for digestion for 15 min at 37°C, and subsequently washed 3 times, according to the specific protocol for this very type of culture.

4. A drop of cell suspension (either trypsinised cell line or trypsin-digested freshly-acquired tissue) is placed on the middle of the coated glass coverslip for a couple of hours and later flooded with media (B27 and Glutamax- supplemented Neurobasal A media for primary brain cultures or DMEM+10% FBS for cell lines or pure astrocytic primary cultures) and kept in an incubator at 37°C/ 5% CO_2. In case of acute tissue slices, the tissue is mounted on the stage of a vibratome with a polyacrylic glue and cut into 100μm sections in frozen cutting solution. After that, the slices are fished and placed in HBSS (Hank's buffered salt solution, Gibco, Cat#14025092) + 10mM HEPES, pH 7.35, or self-made HBSS, composed of 156mM NaCl, 3mM KCl, 2mM $MgSO_4$, 1.25mM KH_2PO_4, 2mM $CaCl_2$, 10mM glucose and 10mM HEPES; pH adjusted to 7.35 with NaOH and supplemented with the fluorescence indicator according to the loading protocol.

3.2 Confocal imaging of mitochondrial ROS production and lipid peroxidation

3.2.1 *Measurements of mitochondrial ROS production with MitoSOX Red*

1. In a 15ml Falcon tube add HBSS buffer, calculated for the number of wells you will measure (1ml /plate), add 5 μM MitoSOX Red, and mix well.

2. Replace the media of the cells with 1ml HBSS buffer containing MitoSOX Red.

3. Leave the cells for 10-15 min at 37°C in a cell culture incubator or at RT/protected from light to load the dye. Loading of this indicator in different tissue and cell types may require amendment of the loading protocol (time, temperature).

4. After 30 min, wash extensively (3x) the fluorescence indicator with RT HBSS and store in HBSS in the incubator till imaging.

5. Mount the glass coverslip, containing the loaded cells to the steel imaging chamber (if not using glass-bottom Petri dish).

6. Clean the coverslip bottom from media residues with a tissue and place the chamber on the objective of the microscope, adding a drop of oil to the objective, if using oil-immersion objective.

7. In phase contrast mode of the microscope, find the region of cells with most appropriate confluence for the experiment.

8. Using the confocal microscope-controlling software, choose appropriate settings for imaging MitoSOX, i.e. excitation using the 514 nm line of the laser, and use a bandpass filter between 550-650nm to collect the emitted light (Abramov et al., 2010).

9. Keep the laser power to a minimum (~1%) and the gain high to ensure appropriate signal while avoiding damaging the living cells.

10. Start a live scan, while adjusting the focus until mitochondria are clearly visible. Stop live scanning.

11. Start the scan (experiment) to obtain the images of MitoSOX Red fluorescence in the chosen regions of interest.

12. Use the recorded table of values for the fluorescence intensity changes over time to build a graph and calculate the slope after linear fitting of the data. The slope will represent the rate of ROS production in mitochondria.

A representative experiment using this method is shown in Figure 1.

Caveat. In certain conditions, we have observed that this ROS indicator loses its mitochondrial tag and accumulates in the nucleus, or in both nucleus and cytoplasm. This is especially problematic for the interpretation of the results in spectrophotometry-based ROS production rate assays or high-throughput screening, without an appropriate thresholding procedure.

3.2.2 *Measurements of mitochondria ROS production with MitoTracker Red CM-H2XRos*

1.In a 15ml Falcon tube add HBSS buffer, calculated for the number of wells you will measure (1ml /plate), add 5 μM MitoTracker Red CM-H2XRos, and mix well.

2. Replace the media of the cells with HBSS buffer containing 100-500 nM (depending on your cell type) MitoTracker Red CM-H2XRos.

3. Leave the cells for 15-45 min (depending on your cell type) at 37°C in a cell culture incubator or at RT/protected from light to load the indicator.

4. After loading time has passed, wash the fluorescence indicator with RT HBSS and store in the incubator till beginning of imaging.

5. Mount the glass coverslip on the steel imaging chamber (if not using glass-bottom petri dish).

6. Clean the glass coverslip bottom from media residues with a tissue and place the chamber on the objective of the microscope, adding a drop of oil to the objective, if using oil-immersion objective.

7. In phase contrast mode of the microscope, find the region of cells with most appropriate confluence for the experiment.

8. Using the confocal microscope-controlling software, choose appropriate imaging settings for imaging MitoTracker Red CM-H2XRos, i.e. excitation using the 514 nm line of the laser, and use a bandpass filter between 550-650 nm to collect the emitted light.

9. Keep the laser power to a minimum (~1%) and the gain high, to ensure appropriate signal while avoiding damaging the living cells.

10. Start a live scan, while adjusting the focus until mitochondria are clearly visible. Stop live scanning.

11. Start the scan (experiment) to obtain the images of MitoTracker Red CM-H2XRos fluorescence in the chosen regions of interest over time.

12. Use the recorded table of values for the fluorescence intensity changes over time to build a graph and calculate the slope after linear fitting of the data. The slope will represent the rate of ROS production in mitochondria.

A representative experiment using this method is shown in Figure 2.

3.2.3 *Measurement of Mitochondrial Lipid Peroxidation with BODIPY™ 581/591 C11*

1. In a 15ml Falcon tube add HBSS buffer, calculated for the number of wells you will measure (1ml /plate), add 1 μM final concentration of BODIPY™ 581/591 C11 (4,4-difluoro-3a,4a diazo-s-indacene), and mix well.

2. Replace the media of the cells with 1ml HBSS buffer containing 1 μM BODIPY™ 581/591 C11.

3. Leave the cells for 30 min (depending on your cell type) at 37°C in a cell culture incubator or at RT/protected from light to load the indicator.

4. After loading time has passed, wash the fluorescence indicator with RT HBSS and store in the incubator till imaging.

5. Mount the glass coverslip, containing the loaded cells, to the steel imaging chamber (if not using glass-bottom petri dish).

6. Clean the coverslip bottom from media residues with a tissue and place the chamber on the objective of the microscope, adding a drop of oil to the objective, if using oil-immersion objective.

7. In phase contrast mode of the microscope, find the region of cells with most appropriate confluence for the experiment.

8. Using the confocal microscope-controlling software, choose appropriate imaging settings for imaging BODIPY™ 581/591 C11, i.e. lipid phase peroxidation is determined by measuring both - the red fluorescence decay of BODIPY™ 581/591 C11, excited at 561nm, and the green fluorescence increase of the oxidation product, excited at 488 nm.

9. Keep the laser power to a minimum (~1%) at the gain high, to ensure appropriate signal while avoiding damaging the living cells.

10. Start a live scan, while adjusting the focus until mitochondria are clearly visible. Stop live scanning.

11. During the scanning replace HBSS in the chamber with "pseudo-intracellular" solution as described above (Materials, 2.8).

12. While visualizing plasma membrane permeabilisation - a decrease of BODIPY™ 581/591 C11 fluorescence intensity from the cytosol for example, and localization of the remaining signal in the mitochondria, wash out the digitonin and replace the buffer with the "pseudo-intracellular" solution.

13. Choose the ROI (region of interest) to be imaged.

14. Start the scan to obtain the images of BODIPY™ 581/591 C11 fluorescence in the cells.

14. Analysis of the images is done by Zeiss software, however, all confocal microscopes are equipped with imaging software that allow for the recording of time course of the fluorescence intensities at the chosen ROI (region of interest). The rate of lipid peroxidation in mitochondria is expressed by the time change (slope of the linear fit) in the fluorescence intensity ratio of green vs. red fluorescence (Ludtmann et al., 2018).

A representative experiment using this method is shown in Figure 3.

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Figure Legends

Figure 1. Time course of the rate of mitochondrial ROS production, imaged with MitoSOX Red

Figure 2. Superoxide generation measurement with MitoTracker Red CM-H2XRos

Figure 3. Time curves of red fluorescence (Ex=561 nm) and green fluorescence (Ex=488nm nm) of C-11-BODIPY in rat mitochondria. C11-BODIPY red fluorescence: □; C11-BODIPY green fluorescence: ◊. Values are mean ± SEM of 3 independent experiments.